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Applications and technical challenges of fluorescence in situ hybridization in stem cell research☆

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Abstract

Stem cell research, maintenance, and manipulations have advanced significantly in recent years, and we now witness successful clinical applications of stem therapies. However, challenges in regard to karyotypic stability and the ploidy status of stem cell lines have been addressed only marginally. Our approach to develop technology to address these highly relevant issues is based on fluorescence in situ hybridization (FISH) using nonisotopically labeled DNA probes. As a single cell analysis technique, FISH is expected to be applicable to a variety of cells and tissues including interphase and metaphase cell preparations as well as tissue sections and biopsy material. Over the last decade, our laboratories generated a large number of probes and probe sets for the molecular cytogenetic analyses of stem cells derived from different species. These probes and the introduction of spectral imaging bring us close to be able to perform a comprehensive karyotype analysis of single interphase cell nuclei. It should furthermore be possible to couple cytogenetic investigations of the cellular genotype with analysis of gene expression. This report summarizes our technical achievements relevant to stem cell research and outlines plans for future research and developments.

Keywords: Stem cells; Cytogenetics; Chromosome enumeration; Fluorescence in situ hybridization; DNA probes; Spectral imaging; Spectral karyotyping

Introduction

Fluorescence in situ hybridization (FISH) is the method of choice for the rapid cytogenetic analysis of interphase and metaphase cells. Based on hybridization of nonisotopically labeled DNA probes to chromosomes, cells, or tissue specimens mounted on microscope slides, FISH allows the analysis of multiple chromosomes or loci in just a few hours. The advantages of FISH over other cytogenetic analysis techniques such as chromosome banding or Barr body analysis become even more obvious when archival tissues are to be examined, the number of cells available for analysis is very limited, or individual cells in heterogeneous mixtures need to be analyzed.

While past applications of FISH have mostly focussed on the characterization of tumor cell specimens, the last decade has seen an increasing use of FISH in prenatal diagnosis, cytogenetic studies in in vitro fertilization (IVF) experiments, and stem cell research, among others. This is because numerical and, to a lesser extent, structural chromosome aberrations exert a negative effect on the proper biological functions of cells. For example, autosomal monomies are incompatible with embryonic or fetal survival, and those trisomies that survive to term (i.e., trisomies 13, 18, or 21) lead to live births with a severely altered phenotype [1].

Carriers of balanced translocations, on the other hand, often experience reproductive problems caused by an ele-

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vated risk of producing aneuploid germ cells due to disturbed homologue pairing in meiosis [2,3]. The resulting aneusomies commonly lead to failed fertilizations and nidation as well as spontaneous abortions. Assisted reproductive technology now offers couples at risk FISH-based approaches to identify unaffected embryos, thus increasing the rates of fertilization and pregnancies as well as reduce the risk of carrying a genetically abnormal fetus [4].

In stem cell research and therapy, FISH-based studies can rapidly detect chromosomal abnormalities or classify cells concerning ploidy or gender. This is expected to facilitate the establishment of stem cell lines suitable for therapeutic applications. Furthermore, the presence or absence of a Y chromosome provides a convenient marker for grafted cells in sex-mismatched transplantations [5–7].

Our laboratories have been involved in the development of nucleic acid hybridization-based procedures for the rapid detection of structural and numerical chromosome abnormalities [3,8–15] as well as the development of Y chromosome-specific probes for different species [16–18]. Since only interphase cells are available for analysis in most instances, our technical developments are geared towards increasing the amount of cytogenetic information that can be obtained in each experiment. One of the goals of our most recent efforts is to provide the tools that enable researchers to perform a full karyotype analysis of single interphase cells. This report attempts to summarize the present state of FISH-based technologies for interphase cell analysis in stem cell research while providing an outlook on new diagnostic technologies that eventually will combine karyotype studies with investigations of gene expression at the single cell level.

Materials and methods

Probe preparation and FISH

We have been involved in the development of probes for FISH analyses for over a decade. Initially, we prepared DNA repeat probes suitable for interphase cell analyses [8–10]. These probes target heterochromatin in the pericentromeric regions of most human autosomes or the X chromosome as well as large clusters of satellite DNA on chromosomes 1, 9, 16, and Y [8–10,16,17]. Table 1 lists the major type of DNA probes used in cytogenetic studies. Major applications of the DNA repeat probes focused on the detection of numerical chromosome aberrations in tumor samples and donor cell detection in sex-mismatched bone marrow transplants or xenografts of retinal pigment epithelial cells [5–7,19].

Since numerical chromosome aberrations are detrimental to early human development and FISH allows the analysis of single cells, the technique rapidly found use in the analysis of blastomeres biopsied from preimplantation embryos of patients enrolled in IVF programs. For example, in collaboration with scientists at the St. Barnabas Medical Center, Livingston, NJ, University of California researchers studied the chromosomal composition of first polar bodies and blastomeres about numerical as well as structural aberrations. In these studies, DNA repeat probes were often used in combination with locus-specific probes (LSPs). The technical aspects of our probe preparation and multicolor detection protocols have been published previously [3,14,16,18,20,21].

Table 1
The three major types of DNA probes applied in FISH analysis

Target	Complexity	Target	Application
DNA repeats	Very low	Heterochromatic regions	Chromosome enumeration
Locus-specific probes (LSP)	Low	Genes, telomeres	Gene mapping, chromosome enumeration
Whole chromosome paints (WCP)	High	Individual chromosomes	Detection of structural abnormalities in metaphase spreads

A major goal of our technical developments is to maximize the number of chromosomal targets that can be scored simultaneously. Recently, we reported the use of a probe set composed of 10 uniquely labeled DNA probes for scoring of chromosomes in interphase cells (Table 2) [22]. Briefly, probes that are specific for repeated DNA on chromosomes 15, X, and Y (Vysis, Inc., Downers Grove, IL) were labeled with either a green or red fluorochrome (Spectrum Green™ or Spectrum Orange™, respectively). The probes specific for chromosome 9, 13, 14, 16, 18, 21, and 22 were prepared in house and labeled by random priming (BioPrime™ Kit, GIBCO/LTI, Gaithersburg, MD) that incorporated biotin-14-dCTP (part of the BioPrime Kit), digoxigenin-11-dUTP (Roche Molecular Biochemicals, Indianapolis, IN), fluorescein-12-dUTP (Roche Molecular Biochemicals), or Cy3-dUTP (Amersham, Arlington Heights, IN). Biotinylated probes were detected with avidin-Cy5 and digoxigenin-labeled probes were detected with Cy5.5-conjugated antibodies against digoxin (Sigma, St. Louis, MO) (Table 2). Between 0.5 and 3 μl of each probe along with 1 μg human COT1™ DNA (GIBCO/LTI) and 20 μl salmon sperm DNA (3′-5′, Boulder, CO) were precipitated with 1 μg glycogen (Roche Molecular Biochemicals) and 1/10 volume of 3M sodium acetate in two volumes of 2-propanol. The precipitate was air dried and resuspended in 3 μl water before 7 μl of hybridization master mix [78.6% formamide (FA, GIBCO/LTI), 14.3% dextran sulfate in 2.9× SSC, pH 7.0 (1× SSC is 150 mM NaCl, 15 mM Na citrate)] were added. This gave a total hybridization mixture of 10 μl.

Metaphase spreads from white blood cells

Metaphase spreads were made from phytohemagglutinin-stimulated short-term cultures of normal male lymphocytes according to the procedure described by Harper and Saun-

Table 2
A 10 probe set for chromosome enumeration in interphase cells using spectral imaging

Target chromosome	Spectrum green	FITC	Spectrum orange	Cy3	Biotin (Cy5)	Digoxigenin (Cy5.5)
9		+				
13		+				+
14		+		+		
15			+			
16				+		+
18				+	+	
21						+
22					+	
X	+		+			
Y	+					

Probes labeled with biotin or digoxigenin were detected with avidin-Cy5 and Cy5.5-conjugated antibodies against biotin or digoxigenin, respectively [22].

ders [23,24]. Fixed cells were dropped on ethanol-cleaned slides in a CDS-5 Cytogenetic Drying Chamber (Thermatron Industries, Inc., Hauppauge, NY) at 25°C with 45% to 50% relative humidity. Slides were stored at room temperature for at least 2 weeks, then in sealed plastic bags under nitrogen gas at −20°C until used.

Blastomeres

All procedures followed protocols approved by the UCSF Committee on Human Research regarding the use of embryos for research. Prior written consent was obtained from all donors. All embryos used for this study either had signs of arrested development or were morphologically abnormal. The zona pellucida of embryos was removed in 0.5% pronase after which the embryo was placed in Ca^{2+}/Mg^{2+}-free PBS until the blastomeres came apart during repeated pipetting of the embryo. Individual blastomeres were incubated in a hypotonic solution of 1% Na-citrate, 6 mg/ml bovine serum albumin in water for 5 min before being placed on microscope slides and fixed with Carnoy's fixative (methanol/acetic acid, 3:1 or 1:1, v/v) [25]. Throughout the procedure, the nucleus was observed in a phase contrast microscope, and following fixation, its location on the slide was marked. The slide was then dehydrated by immersion in 70%, 80%, and 100% ethanol for 2 min each before it was used for FISH or stored at −20°C.

Uncultured human amniocytes

Amniocytes were donated for research under an approved protocol in accordance with guidelines set by the UCSF Committee on Human Research. Uncultured amniocytes were obtained from 1 to 2 ml of amniotic fluid and fixed on slides within a few hours of amniocentesis. The cells were pelleted and then resuspended in a hypotonic solution consisting of 0.3% KCl for 30 min at 37°C. Several drops of ice-cold fixative (methanol/acetic acid, 3:1, v/v) were added and gently mixed, after which the cells were spun down. The cells were resuspended with ice-cold fixative and pelleted several times. Then, the cells were dropped on fixative-cleaned slides above a boiling water bath. The slides were air dried and aged in 2× SSC at 37°C for 1 h. Cells were pretreated with pepsin (50 μg/ml pepsin in 0.01N HCl) at 37°C for 13 min, 1× PBS at room temperature for 5 min, and then postfixed in 1% formaldehyde in 1× PBS/$MgCl_2$ at room temperature for 5 min and washed in PBS. Finally, the slides were dehydrated in 70%, 80%, and 100% ethanol for 2 min each. The slides were used for FISH or stored at −20°C.

Mouse embryonic stem (ES) cells

Metaphase spreads were prepared from cultures of murine AC-4-33 ES cells [26,27]. Briefly, cells were arrested at metaphase with 2 μg/ml colcemid (Sigma) for 1 h and dissociated from the culture flask with trypsin. The cells were then treated with 75 mM KCl solution and fixed with Carnoy's solution. Fixed cells were dropped on ethanol-cleaned slides at room temperature and allowed to age for 3 weeks at room temperature in ambient air. Spectral karyotyping (SKY) of the metaphases was performed with mouse-specific SKY probes as described [28,29].

FISH analysis

All FISH studies were performed following published protocols [3,10,15,16,20,28]. Briefly, slides were denatured in 70 FA, 2× SSC at 76°C for 3 to 5 min, and dehydrated in a 70%, 85%, and 100% ethanol series. The hybridization mixture was denatured at 76°C for 5 min and applied to the slides. A coverslip was placed on top and sealed with rubber cement. Following overnight incubation at 37°C, slides were washed in two changes of 50% FA, 2× SSC at 42°C for 15 min each, then in 2× SSC for twice 15 min. Slides were drained and counterstained with DAPI (Calbiochem, La Jolla, CA).

All SKY experiments followed the procedure recommended by the probe manufacturer (ASI).

Image acquisition

We used a computer-assisted microscopy system for multicolor visualization of probes. The system consists of a fluorescence microscope equipped with a CCD camera, a motorized filter wheel to change excitation filters, and a workstation that controls the filter wheel and camera. The essential optical feature of the microscope is the use of a multiband beam splitter and emission filter. Each fluorochrome in the specimen is excited by selecting the appropriate excitation filter. The band passes in the beam splitter and emission filter are such that all of the fluorochrome-specific images can be obtained without moving any of the elements in the imaging pathway. The current filters (Chro-

maTechnology, Brattleboro, VT) are capable of excitation in single bands centered around 360, 405, 490, 555, and 637 nm and visualization in multiple bands in the vicinities of 460 nm (blue), 520 nm (green), 600 nm (red), and 680 nm (infrared) [20].

Spectral imaging and SKY analyses were performed on an SD200 SpectraCube™ spectral imaging system (Applied Spectral Imaging, Ltd., Migdal Haemek, Israel) [30,31]. The SD200 imaging system attached to a Nikon E600 microscope consisted of an optical head (Sagnac interferometer) coupled to a multiline CCD camera (Hamamatsu, Bridgewater, NJ) to capture images at discrete interferometric steps. The images were stored as a stack in a Pentium 586/300 MHz computer.

Results and discussion

Traditional filter-based microscope systems limit most FISH experiments for interphase analysis to the simultaneous use of no more than 3 to 5 differently labeled probes [32]. This is sufficient to detect structural alterations in interphase and metaphase cells as well as to score a few chromosomes in interphase cells [11,24,32–34].

Figs. 1a–d show examples of single color FISH for the molecular cytogenetic analysis of interphase and metaphase cells. Hybridization of a biotinylated probe specific for the human Y chromosome decorates most of the long arm of this chromosome when detected with avidin-FITC (green) (Fig. 1a) and produces bright signal domains in interphase cell nuclei (Fig. 1b) [16]. Since cells shown in Figs. 1a–d were counterstained with propidium iodide (red), the FISH signals appear yellow due to the overlap of red and green fluorescence. If chromosome-specific DNA probes generate hybridization domains of different size, they can be combined in single color FISH assays. This is illustrated in Fig. 1d, which shows an assay that combines the before mentioned probe for the human Y chromosome with a pericentromeric probe for the human X chromosome (Fig. 1c).

Mouse models have become essential tools in many aspects of genetic, dietary, environmental, and stem cell research. Similar to the human probes described above, we generated probe sets for the analysis of murine tissue samples. Figs. 1e–g illustrate mouse dual color FISH analysis with probes for X and Y chromosomes detected in green, while a probe specific for mouse chromosome 8 was detected in red. Cellular DNA was counterstained with DAPI (blue) (Fig. 1e). Images recorded from the microscope (Fig. 1f) can be digitally processed to increase the signal-to-noise ratio (Fig. 1g). In this example, the green X- and Y-specific signals can be discerned easily by their size difference, while the autosomal probe generated two red hybridization domains representing the two homologues of chromosome 8 in normal C57bl cells.

The analysis of the murine AC-4-33 ES cell line using the triple probe (chromosomes X, Y, and 8), dual color detection scheme revealed the presence of three copies of mouse chromosome 8, one copy of the X chromosome, and no Y chromosome in interphase and metaphase cells (Fig. 1i).

Similar FISH schemes can be applied to the detection of grafted mouse cells in sex-mismatched stem cell transplantations [5–7]. As Fig. 1h shows, male mouse cells transplanted into a female recipient can be detected in liver tissue by the presence of a Y chromosome-specific signal (red).

Spectral imaging (SIm) and spectral karyotyping (SKY)

Presently, commercially available kits allow only up to five chromosomes (X, Y, 13, 18, and 21 or 13, 16, 18, 21, and 22) to be detected simultaneously in interphase cells by using filter-based fluorescence microscope systems (Vysis, Inc.). Our laboratories in collaboration with IVF clinics have developed FISH protocols to determine the number of selected chromosomes in interphase cells or polar bodies and thus to facilitate selection of normal oocytes for IVF or diploid embryos for transfer. In 1998, we reported the simultaneous hybridization of seven chromosome-specific probes to human lymphocytes interphase cells using a SIm system for detection of bound probes [15]. We then extended our probe panel and optimized cell fixation and hybridization protocols for simultaneous enumeration of 10 chromosomes in interphase nuclei using SIm [22,35].

A key element in the proposed technical developments is the application of Fourier spectroscopy-based SIm [30]. SIm combines the techniques of fluorescence microscopy, charge-coupled device (CCD) camera, and Fourier spectroscopy [30]. The light emitted from each point of the sample is collected with the microscope objective and sent to a collimating lens. The collimated light travels through an optical head (interferometer) and is focused on a charged coupled device (CCD). The data are collected and processed with a personal computer. The interferometer divides each incoming beam (the light projected from the microscope) into two coherent beams and creates a variable optical path difference (OPD) between them. The beams are then combined to interfere with each other, and the resulting interference intensity is measured by the CCD detector as a function of the OPD. The intensity vs. OPD is called "an interferogram." The spectrum, that is, intensity as function of wavelength, can be recovered from the interferogram by a relatively simple mathematical operation called "Fourier transformation." This transformation is performed in the personal computer attached to the SIm system. The spectral resolution depends on the number of interferometric steps. For most experiments, a resolution of 10 to 20 nm (equivalent to 64 to 128 steps) is sufficient. Commercially available SIm instrumentation like the Applied Spectral Imaging (ASI, Carlsbad, CA) systems installed in our laboratories can record fluorescence spectra from 400 to 1100 nm with about 10 nm resolution [15,30,31]. The spatial resolution,

limited by the diffraction in the light microscope, is typically better than 500 nm.

First applications of SIm termed "Spectral Karyotyping (SKY)" screened metaphase spreads for translocations [31]. Hybridization of the human chromosomes with 24 chromosome-specific whole chromosome painting (WCP) probes allowed rapid analysis of metaphase spreads in a single experiment. The multiple band pass filter set (ChromaTechnology) used for fluorochrome excitation was custom-designed to provide three broad emission bands centered around 470, 565, and 640 nm. Using a Xenon light source, the spectral image was generated by acquiring 80 to 130 interferometric frames per object. The sample emission spectra (400 to 850 nm) were measured simultaneously at all points in the microscopic image. The spectral information was displayed by assigning red, green, or blue colors to certain ranges of the spectrum. This red, green, and blue (RGB) display renders chromosomes that were labeled with spectrally overlapping fluorochromes or fluorochrome combinations in a similar color. Based on the measurement of the pure spectrum for each chromosome, however, a spectral classification algorithm was applied that allows the assignment of a pseudocolor to all pixels in the image that have the same spectrum. Chromosome identification was then performed by comparison of the measured spectra with prerecorded reference spectra, and chromosomes were displayed in "classification" colors to facilitate the detection of translocations.

The SKY probes are commercially available for the analysis of human or murine metaphase spreads. We applied SKY for characterization of ES cell lines. Figs. 1j and k illustrate the SKY analysis of mouse ES cell line AC-4-33. A spectral image recorded from a metaphase cell (Fig. 1j) was analyzed and individual chromosomes identified based on their fluorescence spectrum. The results were then displayed in the form of an SKY karyotype (Fig. 1k). As indicated by the arrows in Fig. 1k, SKY analysis revealed not only the extra copy of chromosome 8 and the lack of a Y chromosome, but also structural alterations involving chromosome 1 and an extra copy of chromosome 12.

Towards a full karyotype analysis of interphase cells

We explored the use of SIm for chromosome enumeration in interphase cells. The use of whole chromosome-specific painting probes is not possible for interphase analysis with the SIm system because the chromosome domains might overlap (Fig. 1j). Consequently, chromosome-specific DNA repeat or single copy LSPs had to be developed. We constructed a 10-chromosome probe set for detection of DNA targets most frequently associated with aneuploidy and spontaneous abortions (chromosomes 9, 13, 14, 15, 16, 18, 21, 22, X, and Y). Six fluorochromes (spectrum green, FITC, spectrum orange, Cy3, Cy5, and Cy5.5) were used to detect DNA probes. As outlined in Table 2, we scored chromosomes in interphase cells after hybridization with 10 probes labeled with chromosome-specific combinations of the six fluorochromes. For example, a chromosome 9-specific probe was labeled with FITC and a chromosome 18-specific probe was labeled with equal parts of Cy3 and Cy5 (Table 2). The probe set (Table 2) was expected to detect more than 70% of trisomies responsible for spontaneous abortions [1]. We were able to resolve 10 unique chromosome-specific signals in interphase nuclei from different types of cells, including lymphocytes, uncultured amniocytes, and blastomeres [22] (Figs. 1l–p).

For each of these probes, we first acquired a reference spectrum using FISH onto normal metaphase spreads. Following hybridization and the acquisition of spectral images from lymphocytes, blastomeres, and uncultured amniocytes, the reference spectra allowed us to detect and classify chromosome-specific hybridization signals even in the presence of elevated levels of background fluorescence. The results demonstrated the unique power of FISH in conjunction with SIm for identifying chromosomes in single interphase nuclei [22]. Fig. 1l shows an RGB pseudocolored image of an uncultured amniocyte nucleus. The corresponding classification color image of this nucleus is shown in Fig. 1m. The karyotype was that of a normal male cell. Eighteen signals were detected: two copies each of chromosomes 9, 13, 14, 15, 16, 18, 21, and 22 and one copy each of the X and Y chromosomes. Using a separately recorded DAPI image for image segmentation, the SKY software then arranged the chromosome-specific signals as shown in Fig. 1n.

The SIm analysis of blastomeres biopsied from growth arrested human embryos, on the other hand, often demonstrated an abnormal karyotype. As an example, Figs. 1o and p show the RGB picture and the classification color picture, respectively, of a binucleated blastomere. This cell was found to be an abnormal male cell displaying a total of 29 signals. The classification image indicated a large nucleus (Fig. 1o, right) with signals corresponding to two chromosomes 9, four chromosomes 13, four chromosomes 14, two chromosomes 15, two chromosomes 16, five chromosomes 18, three chromosomes 21, two chromosomes 22, and one X chromosome. A smaller nucleus (Fig. 1o, left) contained hybridization targets equivalent to one chromosome 15, one chromosome 18, one X chromosome, and one Y chromosome. Based on the SIm results, this blastomere was considered a hyperdiploid male cell [9(2), 13(4), 14(4), 15(3), 16(2), 18(6), 21(3), 22(2), X(2), and Y(1)].

Based on the results obtained with the 10-probe set, it seems feasible to develop a hybridization scheme to accurately score all chromosomes per cell. The proposed scheme (Table 3) will score eight chromosome types per hybridization and use SIm for detection and signal classification. Following image acquisition, bound probes will be removed and a different set of probes will be hybridized to score a second group of eight chromosome types. By repeating the cycle of probe removal, hybridization, and image acquisi-

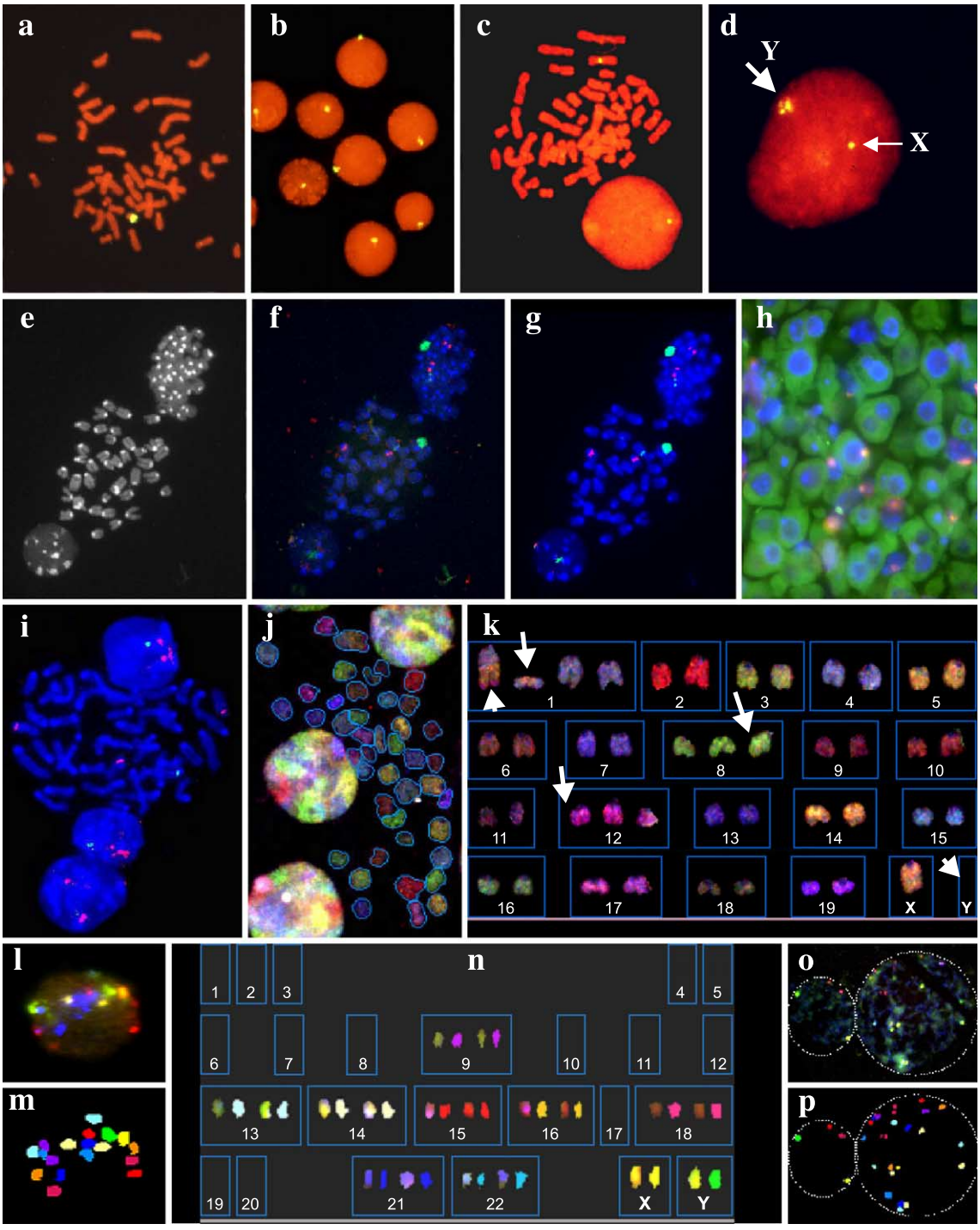

Fig. 1. Hybridization of nonisotopically labeled DNA probes. (a–d) Hybridization of biotinylated probes specific for DNA repeats on the long arm of the human Y chromosome (a and b) or the pericentromeric alpha satellite repeats on the human X chromosome (c). (d) The two probes generate signals of very different size and can be distinguished easily when hybridized simultaneously (d). (e–k) Dual and multicolor FISH for the cytogenetic analysis of murine cells. (e–g) Combined hybridization of probes for mouse chromosomes 8 (red), X (green), and Y (green) to metaphase spreads prepared from bone marrow of a male C57bl mouse. The DAPI image (counterstain) is shown in e. The hybridization results as recorded from the microscope are shown in f and g shows the same image after digital image processing. (h) Male stem cells transplanted into a female recipient can be detected by the presence of a Y-specific hybridization domain (red) (Image courtesy of D. Greer and P. Quesenberry, Center for Stem Cell Biology, Roger Williams Medical Center, Providence, RI). (i) Hybridization of the dual color probe set for mouse chromosomes 8, X, and Y to metaphase spreads prepared from a mouse ES cell line with defective telomeres reveals aneuploidy involving chromosomes 8 and Y. (j–k) Spectral karyotyping analysis of the same ES cell line reveals additional abnormalities involving chromosomes 1 and 12. The arrows point to abnormal, extra, or missing chromosomes. (l–p) Spectral imaging (SIm) analysis of human interphase cell nuclei using a 10-probe set. Following hybridization, signals in uncultured amniocytes are recorded (l) and classified based on their fluorescence spectrum (m). (n) The results are displayed in form of an SIm karyotype. (o and p) SIm analysis of a binucleated human blastomere with the 10-probe set demonstrates an aneuploid karyotype.

Table 3
A proposed scheme for full karyotype analysis using a nine color, eight chromosome enumeration scheme

Target (nm)	Primary label	Secondary label	Excitation (nm)	Emission	Set 1 targets*	Set 2 targets*	Set 3 targets*
Genomic DNA	DAPI	–	363	461			
Target 1	Pacific Blue	–	410	455	13	2	1
Target 2	Spectrum Aqua	–	436	480	14	3	6
Target 3	FITC	–	495	528	15	4	7
Target 4	Cy3	–	552	565	19	5	8
Target 5	Biotin	Cy3.5	581	596	21	12	9
Target 6	Texas Red	–	596	620	22	18	10
Target 7	Cy5	–	650	667	X	11	Y
Target 8	Digoxigenin	Cy5.5	678	703	17	20	16

* The numbers indicate the chromosome type labeled by the hybridized DNA probe.

tion once more with a third set of probes, we will have uniquely marked and recorded all 24 human chromosome types. Challenges that lay ahead are the definition of suitable, minimally overlapping hybridization targets, optimization of cell pretreatment, hybridization, and removal conditions as well as the interactive digital image processing to increase the rate of analysis. The repeated probe stripping and rehybridization, on the other hand, is actually far less challenging than one might think. Similar schemes (although with only 2 to 3 probes) have been used before in preimplantation genetic diagnosis (PGD) and in prenatal analysis [36,37].

Another exciting application of SIm is the quantitative analysis of intracellular levels of different gene transcripts, that is, RNA species. By hybridizing differently labeled cDNA probes, we can uniquely tag individual RNA molecules inside interphase cells to determine their relative amounts. Spectral images can then be analyzed using prerecorded reference spectra and SpectraView software (ASI) to deconvolute the images. The graphic user inter-

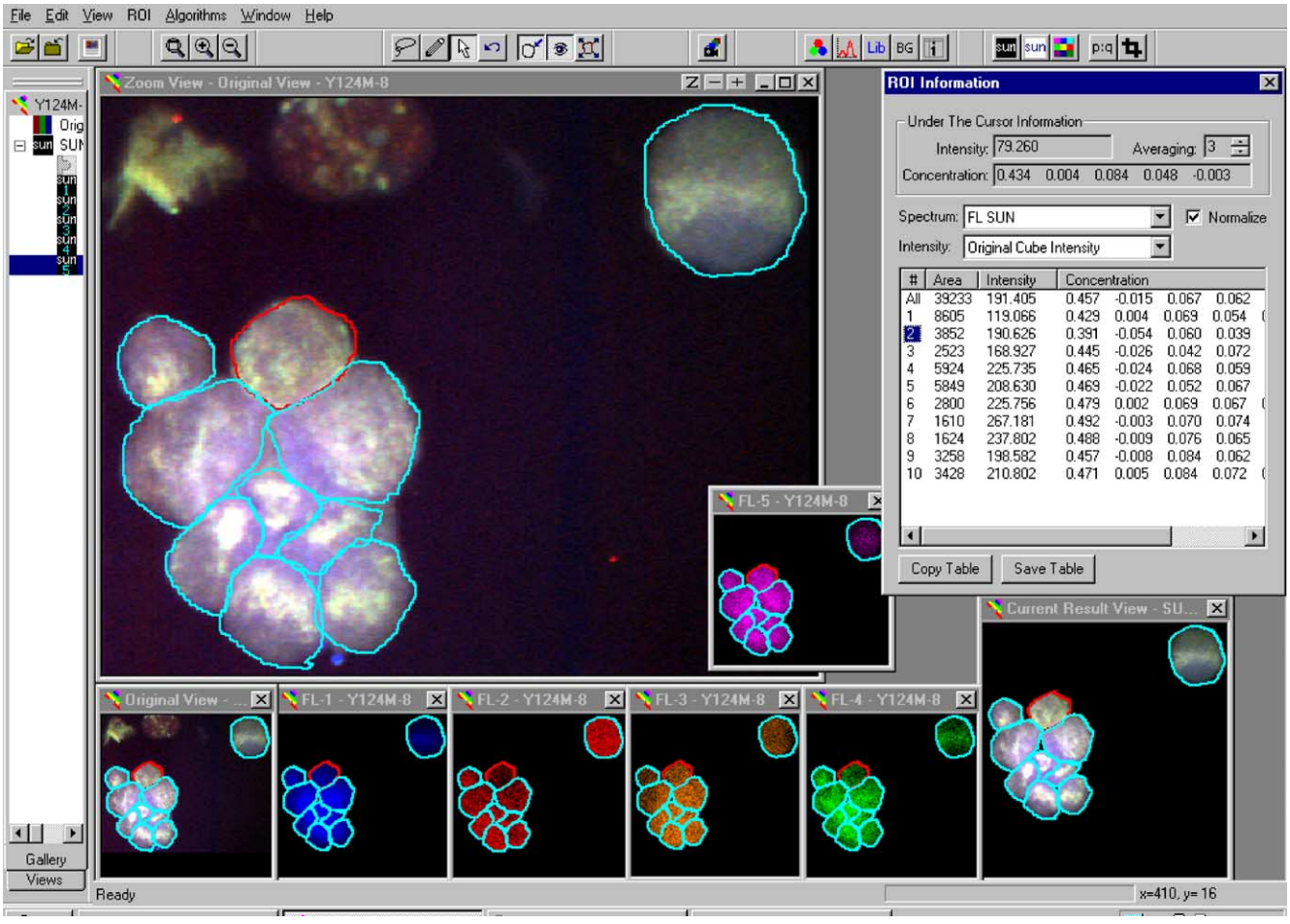

Fig. 2. The graphic user interphase of the SpectraView software (ASI).

phase of the SpectraView software is shown in Fig. 2. The software allows to separate as many as nine colors. The example in Fig. 2 shows the analysis of a spectral image after hybridization of six uniquely labeled cDNA probes specific for different tyrosine kinase genes. The software allows the interactive segmentation of the spectral image, display of single color images representing the major components, and calculation of the relative amount for each color for each region of interest. The numeric data can be imported into spreadsheets to allow comparisons between individual experiments. This may become a powerful tool to survey the expression of stem cell- or tissue type-specific genes in different environments, thus directly addressing issues related to stem cell plasticity.

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